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LXXX. VITAMIN D IN ERGOT OF RYE.

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In the course of experiments carried out with another object it was observed that ergot of rye had a powerful action in promoting calcification of the bones when added to diets which, in themselves, resulted in the development of rickets [Mellanby, Surie and Harrison, 1928]. It was therefore decided to investigate this problem in more detail, to study the properties of the calcifying substance in ergot and to consider its relation to or identity with vitamin D.

Experimental methods.

The animals used in this work were puppies, and the technique was that developed by one of us and described elsewhere [E. Mellanby, 1919, 1921]. It consists essentially in giving young animals of the same litter, from 6 to 8 weeks old, a mixed diet which alone produces rickets. Some are given in addition the substances whose effects on bone development are under investigation and the results on the bones are observed in the test and control animals. Since litters of puppies vary to some extent in their reaction to the basal diet, it is important that in examining the results comparison should be made between the animals in each litter and not between animals in different litters. This caution is also necessary because in the experiments to be described there are variations in the basal diets of different litters so that these diets are sometimes more and sometimes less rickets-producing.

Ergot itself can only be administered to dogs in relatively small quantities owing to its unpleasant taste: even when given in such quantities as from 2 to 5 g. daily the animals often refuse to eat their food, so that it is necessary periodically to omit the substance from the diet. In some experiments the powdered ergot was mixed with bread or meat and administered in pill form in the evening, but even so the food was sometimes refused the following day. In the majority of experiments, therefore, the puppies eating ergot did not grow as well as the other members of the family, a fact which must be taken into account in interpreting nutritional experiments. In spite of these difficulties, however, the antirachitic action of ergot is very evident. When alcoholic and ether extracts of ergot replace the ergot itself the difficulties regarding the food intake are avoided; the animals grow at the same rate as the controls and the results are therefore more satisfactory.

The calcifying action of ergot.

The following sample experiment shows that 2 to 5 g. of ergot added daily to a diet which alone produces rickets assist the calcification processes and tend to prevent rickets.

Exp. 1. Two puppies of one litter were given a rickets-producing basal diet: 1302 received the basal diet only; 1305 was given in addition 2 to 5 g. of powdered ergot.

Age at beginning: 7 weeks.

Duration of experiment: 19 weeks.

Basal diet: 100–160 g. bread, 20 g. lean meat, 20 g. separated milk powder, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 1 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (19 wks.) g.	Gain wt. in 19 wks. g.	Clinical and radiographic diagnosis of bone condition
1302	Basal diet	2400	6985	4585	Bad rickets (Plate II, fig. 1)
1305	Basal diet + 2 to 5 g. of ground ergot	2510	5820	3310	Normal: no rickets (Plate II, fig. 2)

It is evident that the addition of 2 to 5 g. of ergot daily to the basal diet has prevented rickets.

The solubility of the calcifying agent in alcohol.

Exp. 2. 800 g. of ground ergot were extracted twice with 2400 cc. of absolute alcohol. In the first extraction the mixture was boiled for 1 hour and in the second for ¼ hour. The extracts were filtered hot and the alcohol was removed from the combined filtrates under reduced pressure, leaving an oily residue.

Four puppies of the same litter were given a rickets-producing basal diet: 1388 received the basal diet only; 1386 was given in addition the alcoholic extract representing 2 to 4 g. of ergot daily; 1387 was given in addition the residue of ergot after extraction with alcohol, equivalent to 2 to 4 g. of ergot daily; 1389 was given in addition 2 to 4 g. of ergot daily.

Age at beginning: 6 weeks.

Duration of experiment: 11 weeks.

Basal diet: 70–80 g. oatmeal, 20–30 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (11 wks.) g.	Gain wt. in 11 wks. g.	Clinical and radiographic diagnosis of bone condition
1386	Basal diet + alc. ext. of 2 to 4 g. ergot	1725	4720	2995	Rickets (much less than in 1387 and 1388)
1387	Basal diet + ergot residue after alc. extraction	1630	4160	2530	Very bad rickets
1388	Basal diet only	1415	3450	2035	Very bad rickets
1389	Basal diet + 2 to 4 g. ergot	1790	3400	1610	Rickets (much less than in 1387 and 1388)

It is evident that most of the calcifying action of the original ergot is present in the alcoholic extract and that the residue after extraction is devoid of any calcifying effect. The fact that oatmeal forms the cereal in this experiment probably accounts largely for the fact that 1386 and 1389 both have some rickets.

The calcifying effect of the alcohol- and ether-soluble fraction after saponification.

Exp. 3. The oily product obtained from ergot by alcoholic extraction as described in Exp. 2, was well shaken with 500 cc. ether, the insoluble resin being ground up several times with ether to ensure the removal of its ether-soluble contents. The ethereal solution was then filtered and the ether distilled off. This oily residue was dissolved in 100 cc. hot alcohol. 15 g. KOH in 15 cc. water were added and the mixture was boiled under a reflux condenser for 1 hour. The solution was then neutralised with dilute HCl. Portions of this mixture equivalent to 4 g., later to 2 g., of the original ergot were added to the diet of 1407.

Two puppies of the same litter were given a rickets-producing diet: 1406 received the basal diet only; 1407 was given in addition the solution from 2 to 4 g. of ergot after saponification.

Age at beginning: 9 weeks.

Duration of experiment: 10 weeks.

Basal diet: 70 g. oatmeal, 20–30 g. separated milk powder, 20–10 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (10 wks.) g.	Gain wt. in 10 wks. g.	Clinical and radiographic diagnosis of bone condition
1406	Basal diet	2435	4460	2025	Very bad rickets
1407	Basal diet + solution from 4 to 2 g. of ergot after saponification	2145	4320	2175	Almost normal

It is evident that the calcifying factor of ergot is soluble in ordinary ether and is stable to the treatment which saponifies the fats.

Extraction of the unsaponifiable fraction. Having found that the calcifying factor in ergot was ether-soluble and not destroyed by saponification, it remained to see whether it was, like vitamin D, an unsaponifiable substance which could be removed by the ordinary methods for separating this substance from the saponified product.

Our earlier experiments appeared at first to negative this supposition, for the unsaponifiable fraction as then prepared had little or no antirachitic action. The saponification of the oily fraction was carried out as described in Exp. 3. The product was then diluted with its own volume of water and extracted three times with approximately 750 cc. of light petroleum (B.P. 60–80°). The light petroleum was evaporated off and the unsaponifiable residue added to the diet of dogs to test its calcifying properties. Since this is the ordinary method of preparing fat-soluble vitamins from cod-liver oil, it was surprising to find little or no evidence that the calcifying substance had been extracted by the light petroleum.

In subsequent experiments the extraction was made by ordinary ether instead of light petroleum and definite evidence of the presence of the calcifying substance in the unsaponifiable fraction was then obtained, although the activity of the extract did not represent the full activity of the original ergot. Two experiments, one of a preventive and the other of a curative nature, are given as examples to illustrate the calcifying action of the unsaponifiable substance obtained from the fat-soluble fraction of ergot.

The calcifying effect of the unsaponifiable fraction extracted from the saponified fats by ether.

Exp. 4 (*preventive experiment*). Two puppies of the same litter were given a rickets-producing diet: 1497 received the basal diet plus unsaponifiable substance from 4 g. ergot; 1501 received the basal diet only.

Age at beginning: 7 weeks.

Duration of experiment: 12 weeks.

Basal diet: 80–150 g. white flour, 20 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1497	Basal diet + unsapon. subs. from 4 g. ergot	2120	5100	2980	Very little rickets (Plate II, fig. 3)
1501	Basal diet	2650	5820	3170	Very bad rickets (Plate II, fig. 4)

It is clear that the unsaponifiable fraction prepared in this way has prevented in 1497 the very defective calcification seen in 1501.

Exp. 5 (*curative experiment*). Two puppies of the same litter were given a rickets-producing diet. After 10 weeks' feeding, when both had developed very bad rickets, the unsaponifiable substances representing 6 g. of ergot were added daily to the diet of 1517, that of 1519 being continued without alteration.

Age at beginning: 7 weeks.

Duration of experiment: 17 weeks.

Basal diet: 100 to 150 g. bread, 20 g. separated milk powder, 20 to 10 g. lean meat, 10 cc. peanut oil, 2·5 g. dried yeast, 3 cc. orange juice, 1 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (10 wks.) g.	Weight at time of X-ray (17 wks.) g.	Clinical and radiographic diagnosis of bone condition	
					After 10 wks.	After 17 wks.
1517	Basal diet for 10 wks., then unsap. residue from 6 g. ergot for further 7 wks.	2185	4700	5520	Very bad rickets similar to 1519	Healing rickets
1519	Basal diet for 17 wks.	2095	4550	4700	Very bad rickets similar to 1517	Very bad rickets

In this case, the unsaponifiable residue has brought about healing. Puppy 1517 was active to some extent at the end of the experiment, 1519 was paralysed.

Having obtained evidence that the calcifying action of ergot was due to some substance with properties similar to those of vitamin D, we have assumed that the active substance is vitamin D since, so far as is known, this is the only entity which controls the processes of calcification in the body. The problem then arose as to how vitamin D was formed in ergot. Since ergosterol was first discovered as a constituent of ergot by Tanret [1889], and since ergosterol is now known to be transformed into vitamin D by ultra-violet radiation [Rosenheim and Webster, 1927; Hess and Windaus, 1927], the first suggestion as to the mode of origin of vitamin D in ergot was that it was

produced at some stage in the growth of the infected rye by the direct action of the ultra-violet radiations of sunlight on ergosterol. This mode of origin did not, however, appear very probable because ergot of rye has a dark bluish-black covering and there was doubt as to whether this covering was permeable to the radiations. If it were permeable, then, since ergot contains abundant unactivated ergosterol, exposure of the ergot grains to sunshine ought to increase its calcifying activity. In order to test this point the following experiment was carried out.

The effect of irradiating ergot with sunlight and mercury-vapour light.

Exp. 6. A sample of whole ergot grains was scattered sparsely over a tin dish and exposed to bright summer sunshine for 12 hours. Another sample was placed on a similar dish and exposed to a mercury-vapour lamp at a distance of about 2 ft. for half an hour. Each sample was then ground and extracted with alcohol. The alcohol was distilled off from each under diminished pressure and the residues were administered to two dogs on an otherwise poor calcifying diet. The third dog received the alcoholic extract of 2 g. untreated ergot.

Three puppies of the same litter were given a rickets-producing diet: 1498 received the basal diet plus alcoholic extract of ergot irradiated by sunlight; 1499 received the basal diet plus alcoholic extract of ergot irradiated by mercury-vapour light; 1496 received the basal diet plus alcoholic extract of untreated ergot.

Age at beginning: 7 weeks.

Duration of experiment: 12 weeks.

Basal diet: 80–150 g. white flour, 20 g. separated milk powder, 20 g. lean meat, 10 cc. olive oil, 2·5 g. dried yeast, 3 cc. orange juice, 2–3 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1496	Basal diet + untreated ergot (alc. ext. 2 g.)	2430	5300	2870	Rickets similar to 1498
1498	Basal diet + sunlight ergot (alc. ext. 2 g. ergot)	2400	5500	3100	Rickets similar to 1496
1499	Basal diet + Hg-vapour light ergot (alc. ext. 2 g. ergot)	2150	5500	3350	Slight rickets, definitely less than 1496 and 1498

The alcoholic extract of 2 g. only of non-irradiated ergot was given to 1496 so that some degree of rickets should develop, thus allowing the detection of an increase (if any) in the vitamin D after irradiating ergot by sunlight (1498) and by the mercury-vapour lamp (1499). The results of the experiment show (1) that exposure of intact ergot grains to strong sunlight for 12 hours does not increase their calcifying activity and (2) that exposure to the mercury-vapour lamp for half an hour slightly increases the activity. It is evident that the covering of the ergot grains is relatively impermeable even to very abundant ultra-violet radiations. This result raised some doubt as to whether the presence of vitamin D was due to the direct action of sunlight on the ergosterol present in the ergot.

It appeared desirable, then, to decide whether the vitamin D is primarily a product of the sclerotium or whether the rye embryo in which the fungus

develops is responsible to any extent for the presence of the vitamin. If the fungus and its sclerotium were the responsible agent, there was a possibility that other types of fungi would contain vitamin D. To test this point an experiment was made to see whether the ordinary edible mushroom (*Agaricus campestris*) had any calcifying action. It may be mentioned that Gérard [1892, 1895] found ergosterol not only in ergot of rye but in the whole group of Cryptogams—Basidiomycetes, Myxomycetes, Ascomycetes, Oomycetes and lichens. It seemed possible, therefore, that mushrooms might contain the calcifying vitamin. In the one experiment carried out this did not prove to be the case.

In view of the negative result we then turned our attention to rye germ. The specimens used, so far as we know, were not ergotised. Two samples were tested, one obtained from millers in Belgium, and the other from Germany. Both samples looked uniform in composition and microscopic examination did not reveal any evidence of the presence of mycelial threads. In the following experiment, which shows that rye germ itself has slight calcifying properties, the germ used was of German origin.

The effect of rye germ on calcification.

Exp. 7. Four puppies of the same litter were given a rickets-producing diet, in addition to which 1505 received white flour only; 1506 received a mixture of 80 % white flour and 20 % rye germ; 1510 received 80 % white flour and 20 % rye germ which had been extracted with light petroleum; 1504 received 100 % white flour and the light petroleum extract of rye germ equivalent to that used for 1506.

Age at beginning: 6 weeks.

Duration of experiment: 12 weeks.

Basal diet: 25 g. separated milk powder, 20 g. lean meat, 10 cc. peanut oil, 2·5 g. dried yeast, 3 cc. orange juice, 2 g. sodium chloride.

No. of animal	Variations in diet	Initial weight g.	Weight at time of X-ray (12 wks.) g.	Gain wt. in 12 wks. g.	Clinical and radiographic diagnosis of bone condition
1504	White flour 100 % + light petroleum ext. of rye germ	2625	6220	3595	Slight rickets, less than 1505
1505	White flour 100 %	2650	6250	3600	Rickets
1506	White flour 80 % + rye germ 20 %	2585	6320	3735	Rickets, rather more than 1505
1510	White flour 80 % + rye germ after extraction with light petroleum 20 %	2505	6200	3695	Bad rickets: more than 1506

This experiment shows that the light petroleum extract of rye germ contains some calcifying factor. It will also be noticed that rye germ itself does not bring about the improvement in calcification produced by its extract. The reason for this is seen in animal 1510, for, in this case, when the fraction of the rye germ soluble in light petroleum has been removed, the residue actually makes the condition of the bones worse. In other words, rye germ,

Experiment 1

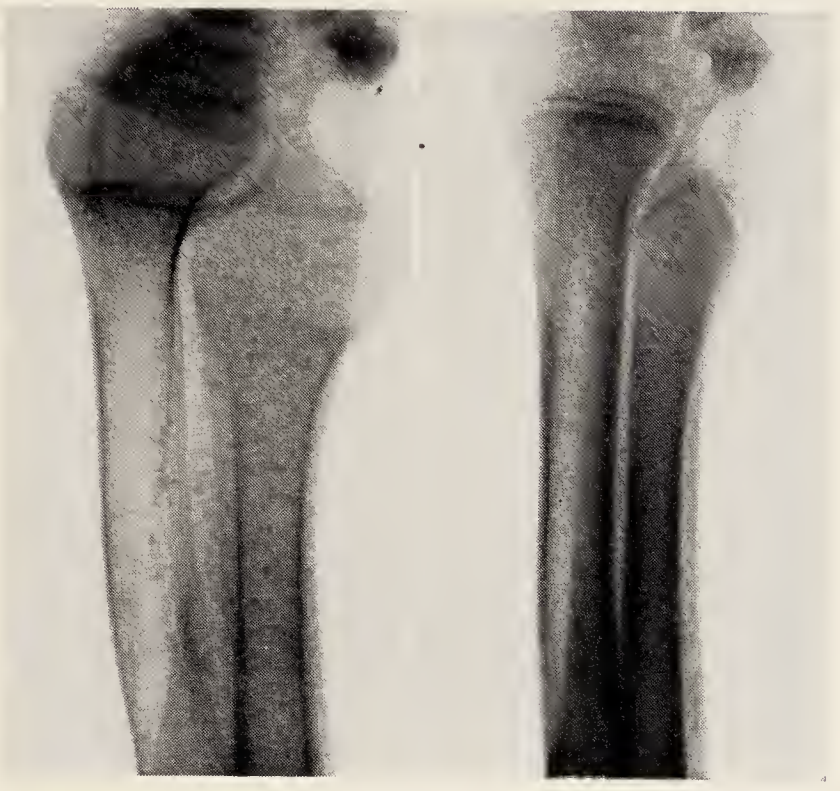

Fig. 1. Fig. 2.

Experiment 4

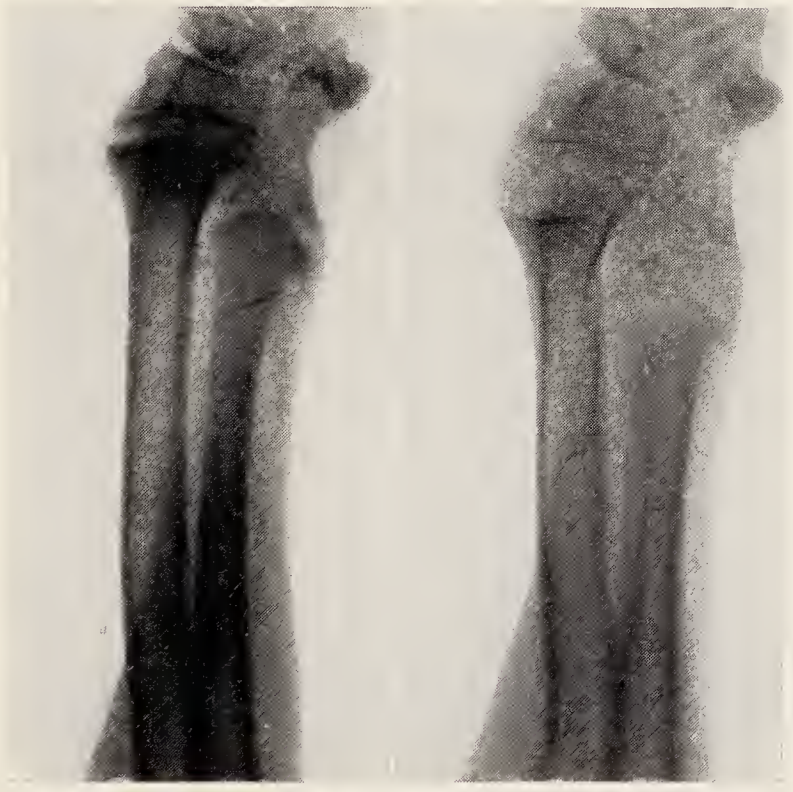

Fig. 3. Fig. 4.

Fig. 1. *Exp.* 1 (1302). Basal diet only. Severe riekets after 19 weeks of diet.
Fig. 2. *Exp.* 1 (1305). Basal diet + 2 to 5 g. ergot. Bones normal.
Fig. 3. *Exp.* 4 (1497). Basal diet + unsaponifiable substances of the fat-soluble fraetion of 4 g. ergot. Very little riekets after 12 weeks of diet.
Fig. 4. *Exp.* 4 (1501). Basal diet. Very bad rickets after 12 weeks of diet.

as has been previously shown by E. Mellanby to be the case with wheat germ, contains a rickets-producing factor.

The following facts therefore must be taken into account when considering the origin of the vitamin D in ergot.

(1) Rye germ and fungi in general contain abundant ergosterol.

(2) Rye germ, unaffected by ergot, contains a small amount of activated ergosterol (vitamin D).

(3) In the one form of fungus tested (mushrooms) there was no evidence of vitamin D.

(4) There is no evidence that the ergosterol in ergotised rye germ can be activated to vitamin D on exposure of the whole grain to direct sunlight.

The fact that the experiment with mushrooms gave no evidence of the presence of vitamin D indicates that abundant ergosterol can be present in fungi without any of it being in the form of vitamin D. This is also true of some of the cereals, including oatmeal. On the other hand, although Exp. 7 demonstrates the presence of vitamin D in normal rye germ, the amount present is small as compared with that in the ergot. This suggests that some of the ergosterol in the fungus *Claviceps purpurea* (ergot) is in the active form. How this ergosterol becomes activated to vitamin D is an unsolved problem. Although at the present time the ultra-violet irradiation of ergosterol is the only known mode of origin of vitamin D, it is possible that vitamin D can be made from ergosterol by the growing plant independently of ultra-violet radiations.

Since vitamin D is found so rarely in nature in any quantity, it is interesting that ergot should be one of its sources. So far as is known, fish and egg-yolk are the only substances which are powerfully calcifying in their action, other substances like milk, animal fats and meat containing smaller quantities of vitamin D. Some samples of ergot may be roughly estimated to contain about one-eighth to one-quarter the calcifying activity of cod-liver oil. All samples of ergot so far tested, whether of Russian or Spanish origin, are definitely antirachitic.

Summary.

1. Ergot of rye is a powerful stimulus to calcification of bone. The factor responsible for this action is soluble in alcohol and ether, resists saponification and has the properties, so far as these are known, of vitamin D.

2. Rye germ itself, unaffected by the ergot fungus *Claviceps purpurea*, contains a small quantity of calcifying substance which can be extracted by light petroleum.

3. The irradiation of unground ergot grains by strong sunlight produces no increase and the irradiation by the mercury-vapour lamp only a slight increase in the calcifying activity of ergot, although there is abundant ergosterol present.

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